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Transcriptional Control of Adrenal Steroidogenesis

NOVEL CONNECTION BETWEEN JANUS KINASE (JAK) 2 PROTEIN AND PROTEIN KINASE A (PKA) THROUGH STABILIZATION OF cAMP RESPONSE ELEMENT-BINDING PROTEIN (CREB) TRANSCRIPTION FACTOR[*][S]

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In the adrenal gland, adrenocorticotropin (ACTH) acting through the cAMP protein kinase (PKA) transduction pathway is the main regulator of genes involved in glucocorticoid synthesis. The prolactin (PRL) receptor is expressed in the adrenal cortex of most mammals, but experimental proof that PRL ensures direct control on glucocorticoid synthesis in rodents remains elusive. To unravel the physiological importance of PRL in adrenocortical functions, we measured steroidogenic capacity of *Prlr*-deficient mice (*Prlr*$^{-/-}$) and explored the influence of JAK/STAT signaling, the major PRL transduction pathway, on the steroidogenic activity of adrenocortical cell cultures. We demonstrate that lack of *Prlr* does not affect basal (nor stress-induced) corticosterone levels in mice. PRL triggers JAK2/STAT5-dependent transcription in adrenal cells, but this does not influence corticosterone release. In contrast, pharmacological or siRNA-mediated inhibition of JAK2 reveals its essential role in both basal and ACTH/cAMP-induced steroidogenesis. We demonstrate that nuclear JAK2 regulates the amount of active transcription factor CREB (cAMP response element-binding protein) through tyrosine phosphorylation and prevention of proteasomal degradation, which in turn leads to transcriptional activation of the rate-limiting steroidogenic *Star* gene. Hence, we describe a novel link between PKA and JAK2 by which nuclear JAK2 signaling controls adrenal steroidogenesis by increasing the stability of CREB.

Acute and chronic adrenal cortex steroidogenesis is regulated mainly through the adrenocorticotropin (ACTH) activation of the cAMP-dependent protein kinase A (PKA) signaling pathway. Chronic response to ACTH results in increased transcription of genes encoding steroidogenic enzymes and the proteins responsible for cholesterol mobilization and transport, such as the steroidogenic acute regulatory protein (STAR),[4] which mediates the rate-limiting and regulated step in steroidogenesis. cAMP-induced PKA activations result at least in the phosphorylation of the transcription factors GATA-4-binding protein, steroidogenic factor 1 (SF1), CAAT enhancer-binding protein (C/EBP) and cAMP response element-binding protein (CREB). These in turn stimulate transcription of genes involved in steroidogenesis (1, 2). The cAMP-responsive unit of the mouse *Star* proximal promoter (−110 and −67 bp) is a prototypical sequence that involves multiple DNA binding motifs for these transcription factors including three CRE half-sites that can bind CREB (2–4).

Although cAMP is an essential inducer of steroidogenesis, other mechanisms triggered by paracrine and endocrine signals have been involved in the regulation of steroid synthesis in basal or stress-induced conditions. They can either act independently or in synergy with the PKA signaling (5). Prolactin (PRL) is a stress-related protein that has been reported to enhance aldosterone and glucocorticoid production in various mammalian species including rat, guinea pig, mouse, pig, and human (6–12). A direct action of PRL on adrenal function has been speculated, on the basis of the PRL receptor (PRLR) expression in adrenocortical cells (12, 13). An indirect action of PRL via increased luteinizing hormone (LH) receptor expression in mouse adrenal cortex has also been reported (14). Downstream from the PRL receptor, a class 1 cytokine receptor member, the Janus kinase-signal transducer and activator of transcription (JAK/STAT) signaling is the best characterized of the PRL transduction pathways. Through the binding to its membrane receptor, PRL induces activating changes in the intracellular domain of the PRLR. These trigger autophosphorylation of the JAK2 kinase, phosphorylation of the PRLR and recruitment and phosphorylation of STAT5a/b. Activated STAT proteins homo- or heterodimerize and translocate to the nucleus where they bind to the γ-interferon-activated site element to promote

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4 and Table S1.

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[4] The abbreviations used are: STAR, steroidogenic acute regulatory protein; 8-Br-cAMP, 8-bromo-cyclic AMP; ca, constitutively active; CRE, cAMP response element; CREB, CRE-binding protein; JAK, Janus kinase; LH, luteinizing hormone; LHRE, lactogen hormone response element; luc, luciferase; PRL, prolactin; PRLR, PRL receptor; RIA, radioimmune assay; SF1, steroidogenic factor 1; tk, thymidine kinase.

transcription of target genes (15, 16). Alternatively, once activated, JAK also phosphorylates various cytokine and growth factor receptors and signaling molecules such as those regulated by the Src kinase, the Ras/MAPK pathway, and the PI3K pathway (17). Recent data obtained in mammary tumor cells demonstrated a new role for JAK2 in the nucleus in preventing proteosomal degradation of transcription factor (18). In the steroidogenic tissues, JAK2 was proposed to act in hormonal signaling triggering increased steroidogenesis. Indeed, JAK2 was shown to be involved in the angiotensin-dependent stimulation of *Star* transcription and progesterone production in the H295R cell line. However the molecular targets of JAK2 remained unidentified in this process (19). More recently, bromocriptine-induced hypoprolactinemia was shown to decrease the stressed induced STAT5 phosphorylation in adrenal gland in Hatano high avoidance rats that display hyperreactivity to stress. These data suggest that PRL acts on the adrenal cortex to regulate adrenocortical function even though the molecular link between PRL/STAT5 and steroidogenic genes has not yet been established (20). Here, using mutant *Prlr* mice and both cell lines and adrenocortical primary culture, we show that PRL has no significant effect on adrenal steroidogenesis *in vivo* and *in vitro*. However our data demonstrate that JAK2 controls adrenal steroidogenesis. This activity relies on a cross-talk between JAK2 and the PKA signaling pathway through control of the stability of the activated CREB protein.

EXPERIMENTAL PROCEDURES

Chemicals—ACTH (fragment(1–24)), 8-Br-cAMP, purified ovine PRL, recombinant ovine PRL, forskolin, H89, PD98059, leptomycin B, and MG132 were purchased from Sigma-Aldrich. Tyrphostin AG490 was purchased from Calbiochem. Recombinant human PRL was kindly provided by Dr. Vincent Goffin (INSERM, Paris).

Experimental Animals—Five-month-old wild-type and $Prlr^{-/-}$ male mice (129/Sv) were generated by crossing heterozygous mice (21). The progeny was classified by PCR analysis of DNA extracted from tails clipping as described previously (22). Mice were housed under normal laboratory conditions in a 12-h day/night cycle at 21 °C and relative humidity 50% with food and water *ad libitum*. Restraint experiments were carried out in a 50-ml conical tube for 30 min before euthanasia. For food deprivation experiments, $Prlr^{+/+}$ and $Prlr^{-/-}$ mice were divided randomly into fed and 24-h fasted groups. Lipopolysaccharide (*Escherichia coli* 0127:B8, Sigma) in 2% FCS was injected intraperitoneal at a dose of 1 mg/kg at 2 p.m., 6 h before euthanasia. Animals were killed by decapitation, and trunk blood was collected in 0.5 M EDTA-treated tubes and followed by centrifugation. Plasma was separated and stored at −80 °C until assayed for ACTH and corticosterone. Experimental procedures were in agreement with the guidelines of the animal ethic committee of the ministère de l'agriculture.

Adrenal Cell Cultures—Eight-week-old male Wistar rats weighing 300–350 g were killed by CO_2 anesthesia. Adrenals were rapidly removed, and the medulla was separated from the cortex by squeezing the gland after making an incision through the capsula. Adrenal cortex were cut into three or four pieces. Tissue fragments were incubated in Hanks' balanced saline solution for 20 min at 37 °C in the presence of collagenase type I (1 mg/ml) (Sigma-Aldrich). Dispersed cells were pelleted, and the remaining tissues were submitted to three or four repeated collagenase incubations until digestion was complete. Supernatant of cortical viable cells was pooled and seeded at the density of 400,000 cells/well in polylysine-coated 6-well plates and maintained at 37 °C, 5% CO_2 in DMEM-Ham's F12 medium (DF12) containing 100 units/ml penicillin and 100 μg/ml streptomycin supplemented with 2.5% calf serum, 2.5% horse serum, insulin (10 μg/ml), transferrin (5.5 μg/ml), selenium (6.7 μg/ml) (ITS; Invitrogen) for 2 days. Cells were starved for 24 h in minimum medium (without serum) before the addition of hormones and reagents at times and concentration indicated in the figure legends. The murine ATC1 cell line established from adrenocortical tumors of transgenic mice harboring the Large T antigen of SV40 under the control of the adrenocortical specific promoter of *Akr1b7* gene (23) was grown under the same conditions as described for rat primary culture.

Hormonal Assays—Corticosterone released in culture medium was determined by RIA using a commercially available kit (ICN Biomedicals). ACTH dosage in plasma or in various sources of PRL (purified from pituitary or recombinant) were performed by solid phase, two-site sequential chemiluminescent immunometric assay (Siemens Healthcare Diagnostic SAS, Saint-Denis, France) using an Immulite 2000 analyzer.

cAMP Assays—Corticosterone from murine plasma or released in culture media were determined by RIA using a commercially available kit (ICN Biomedicals). ACTH from murine plasma was determined by ELISA in the biological laboratory of Clermont-Ferrand Hospital (CHRU). An intracellular cAMP assay was performed using a sensitive protein-binding enzymoimmunoassay kit (Cayman Chemicals). For this assay, ATC1 cells were plated at a density of 400,000 cells in 6-well dishes in Complete medium. After 24 h, the cells were incubated for 1 h at 37 °C in serum-free medium containing 0.5 mM 3 isobutyl-1-methylxanthine (Sigma-Aldrich) and treated for 20 min with 10^{-9} M ACTH. Additional inhibitor AG490 and was added 7 h before treatment with ACTH. The reaction was stopped by adding 0.1 N HCl and incubated at room temperature for 20 min. The cells were scraped and transferred to a 1.5-ml tube and centrifuged for 10 min at 1000 × *g*. The supernatant was used to assay for cAMP.

RT-PCR—Total RNA was isolated from ATC1 cells and rat adrenal primary culture with TRIzol (Invitrogen) according to the manufacturer's instructions and reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Promega) and amplified using TaqDNA polymerase (Promega) as described previously (23). Nucleotide sequences of the specific primers used were: *Prlr* forward, 5′-GAGAAAAACACCTATGAATGTC-3′ and reverse 5′-AGCAGTTCTTCAGACTTCC-3′; β-actin forward, 5′-CGTGGGCCGCCCTAGGCACCA-3′ and reverse, 5′-TTGGCCTTAGGGTTCAGGGGGG-3′. The primer pairs yielded PCR products of 647 bp and 242 bp for *Prlr* and β-actin, respectively. PCR consisted of 20 cycles of the following steps: denaturation for 45 s at 94 °C, annealing for 45 s at 54 °C, extension for 45 s at 72 °C. PCR product was resolved on a 1.5% agarose gel.

Cell Transfection—Transfections in ATC1 cells were conducted in 6-well plates using Metafectene reagent (Biontex Laboratories GmBH) according to the manufacturer's instructions. ATC1 cells were seeded at a density of 400,000 cells/well and transfected in serum-free medium for 12 h and then incubated with 10^{-7} M ACTH or 1 mM 8-Br-cAMP in the absence or in presence of 30 μM AG490 JAK2 inhibitor reagent for time indicated in the figure legends. The *luc* reporter constructs transfected in ATC1 cells were driven either by murine *Star*, rat somatostatin regulatory regions, or a construct containing six STAT5a binding sites upstream to the minimal thymidine kinase gene promoter: −966 *Star-luc* and −113 *Star-luc* were provided by Dr. D. Mangelsdorf (University of Southwestern Medical Center, Dallas, TX), −65 somatostatin-luc containing a cAMP response element in −48 bp was provided by Prof. J. Bertherat (Institut Cochin, Paris). A construct containing six binding sites for STAT5 associated with the minimal thymidine kinase promoter was named LHRE (lactogen hormone response element) *tk luc* (24). In co-transfection experiments, 250 ng of either plasmid encoding the constitutively active STAT5 a mutant under the control of RSV promoter (25) or corresponding pRSV empty vector was used with 2 μg of reporter gene and 2 ng of pRLSV40 (Promega). Luciferase and *Renilla* assays were performed as described previously (26) in an automated luminometer with Genofax A and Genofax B reagents (Yelen). Each experiment was performed in quadruplicate and repeated at least three times. All data are expressed as means ± S.D.

RNA Interference—A 21-nucleotide small interfering RNA (siRNA) duplex targeting mouse JAK2 RNA was custom synthesized by Dharmacon. The antisense oligonucleotide as ordered was 5′P-GAAACUUCAGAUACUCUCCUU with 3′ UU overhangs and targets the sequence GGAGAGUAUCUGAAGUUUC corresponding to nucleotides 247–265 at the N-terminal region of the JAK2 protein (amino acids 52–58). The control nontargeting siRNA (UAGCGACUAAACACAUCAA) with 3′UU overhang was obtained from Dharmacon. Transfection of siRNA duplexes was carried out using Metafectene (Biontex) according to the manufacturer's instructions. Two days after transfection, medium was collected for corticosterone assay, and the cells were harvested for cellular extract preparation or luciferase assay. RNA interference using a CREB siRNA previously described (27) was performed for gene reporter studies in ATC1. Per well, 200 nM CREB siRNA or Scramble siRNA was transfected for 24 h prior to transfection with 2 μg of reporter gene. Twelve hours after transfection, cells were treated with 10^{-9} M ACTH for 16 h, and luciferase assays were performed as described below. For STAT5a knockdown, 200 nM STAT5 siRNA or Scramble (Cell Signaling) was transfected for 36 h prior to transfection with 2 μg of reporter gene. Six hours after reporter gene transfection, cells were treated for 12 h (or 30 min for Western blot analysis) without human recombinant 10^{-8} M PRL. Each experiment was performed in triplicate and repeated at least three times. All data are expressed as means ± S.D.

Cellular Extracts and Immunoprecipitation—Cell samples were homogenized and incubated for 20 min in ice-cold extraction buffer containing 50 mM Tris (pH 7.5) 300 mM NaCl, 10 mM $MgCl_2$, 0.4% Nonidet P-40, 1 mM NaF, 1 mM VO_4Na_3, 1 mM DTT, and protease inhibitors mixture (Complete protease inhibitor mixture tablets; Roche Diagnostics). After centrifugation for 15 min at 2000 rpm, the concentration of soluble proteins was determined by the Bradford method (Bio-Rad). Then 800 μg of proteins from the supernatant were used for immunoprecipitation with anti-CREB (Cell Signaling) and anti-JAK2 (Upstate) antibodies and protein A-Sepharose (Amersham Biosciences) before Laemmli sample buffer treatment.

Subcellular Fractionation—ATC1 cells (5×10^7 cells) were scraped gently in PBS-10% glycerol (v/v) and washed in a hypotonic buffer A (50 mM NaCl, 20 mM HEPES (pH 7), 1 mM EDTA, 0.25 mM EGTA, 0.5 M sucrose) containing 1× protease inhibitor mixture (Complete), 1 mM NaF, and 1 mM VO_4Na_3. They were then resuspended and incubated on ice for 30 min in buffer A plus 0.5% Nonidet P-40. Centrifugation at 5000 rpm for 10 min was performed to separate nuclei from the other remaining cellular compartments (supernatant). The supernatant was collected, and nuclei were washed four times with the Complete hypotonic buffer and then were lysed by homogenization and incubation in hypertonic buffer (buffer A with 0.45 M NaCl) for 10 min at 4 °C. Centrifugation at 12,000 rpm for 20 min was then performed to isolate nuclear soluble proteins from chromatin. Thirty μg of total proteins from whole cell extracts or nuclear or supernatant fraction were analyzed by Western blotting.

Western Blot Analysis—Western blotting was performed as described previously (23). Blots were incubated at 4 °C with primary polyclonal antibodies against Sr-b1 (gift from C. Sérougne, Université Paris-Sud, 1/2000[e]), JAK2 (Cell Signaling, 1/1000[e]), Phospho-JAK2 (Tyr^{1007}/Tyr^{1008}; Cell Signaling, 1/1000[e]), CREB (Cell Signaling, 1/1000[e]), Phospho-CREB (Ser^{133}) (Cell Signaling, 1/1000[e]), SF1 (1/2000[e]) (28), PKA Cα unit (Cell signaling, 1/1000[e]), STAR (1/5000[e]) (29), and β-actin (Sigma Aldrich, 1/3000[e]) or monoclonal phosphotyrosine Py (Upstate, 1/1000[e]). Primary antibodies were detected with a secondary antibody conjugated to peroxidase (PARIS). Peroxidase activity was detected with the enhanced chemoluminescence system (ECL, PerkinElmer Life Sciences).

Statistical Analysis—For comparison between two groups, the unpaired two-tailed Student's *t* test was performed. One-way ANOVA followed by the Student-Newman-Keuls procedure was used to compare more than two groups.

RESULTS

JAK2 but Not PRL Controls Corticosterone Production—Transduction of prolactin signaling involves a specific membrane receptor (PRLR) expressed in adrenal cortex (12). Consequences of the genetic ablation of the PRLR on the pituitary-adrenal axis were analyzed by measuring morning (8:00 a.m.) and evening (5:00 p.m.) plasma corticosterone and ACTH concentrations in PRLR-deficient mice ($Prlr^{-/-}$) compared with wild-type males. These *in vivo* experiments showed no differences between $Prlr^{+/+}$ and $Prlr^{-/-}$ males for plasma levels of corticosterone (17.2 ± 20.4 ng/ml *versus* 21.7 ± 13.6 ng/ml at 8:00 a.m. and 32.5 ± 14.7 ng/ml *versus* 41.8 ± 10.9 ng/ml at 5:00 p.m.) and ACTH (331 ± 131 pg/ml *versus* 354 ± 156 pg/ml at 8:00 a.m. and 518 ± 141 pg/ml *versus* 565 ± 110

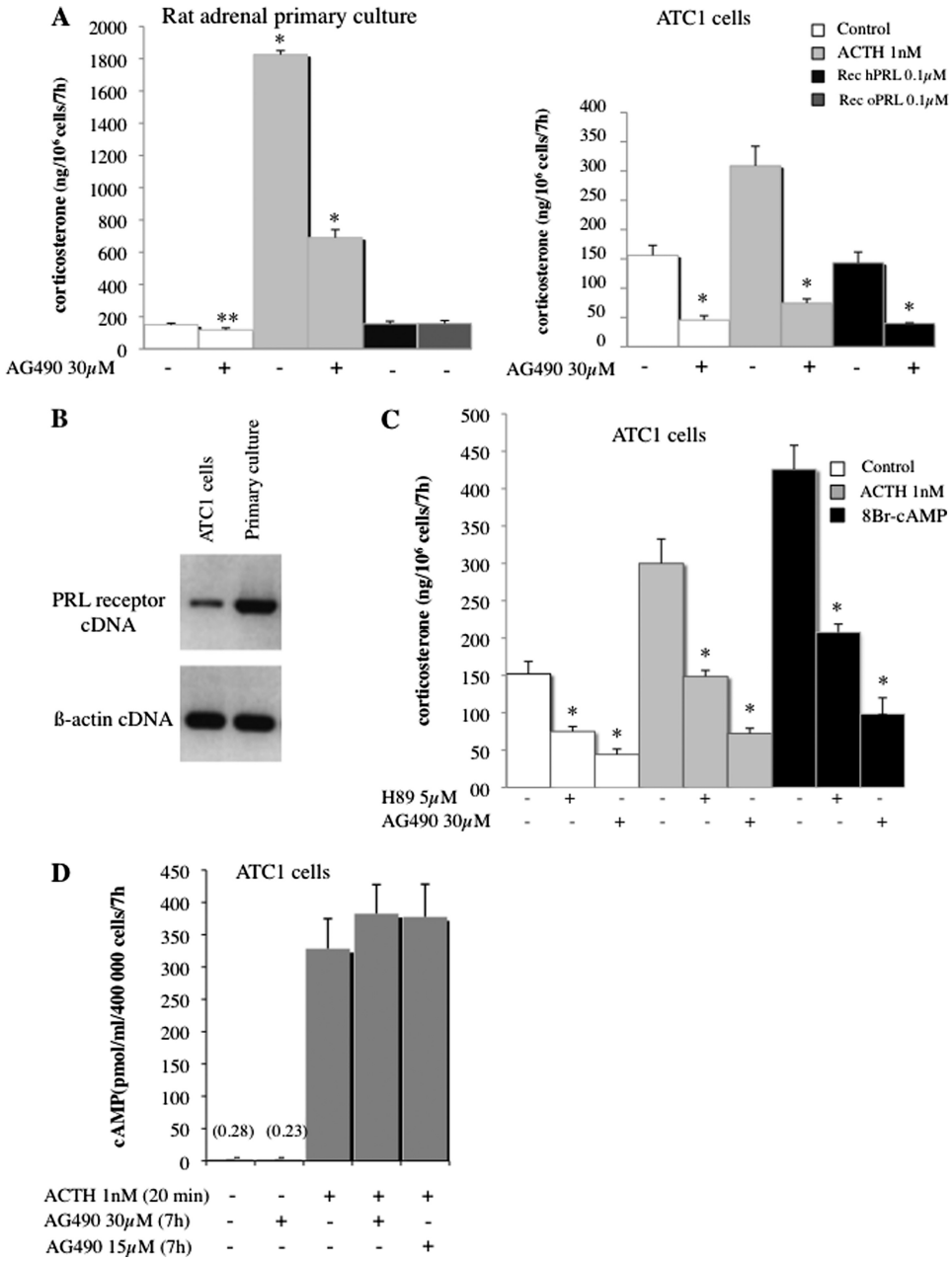

FIGURE 1. **Corticosterone production is controlled by JAK2 under basal and ACTH conditions.** *A* and *C*, rat adrenal primary culture (*A*) or ATC1 cells (*A* and *C*) were incubated for 7 h with (+) or without (−) the AG490 JAK2 inhibitor or the H89 PKA inhibitor in presence of ACTH or human or ovine recombinant PRL for the last 6 h. Corticosterone productions in culture medium were assayed by RIA. Each value represents the mean of six independent experiments ± S.D. (*error bars*) (*, $p < 0.05$; **, $p < 0.1$ compared with dimethyl sulfoxide- (inhibitor-free) treated cells under respective hormonal condition). *B*, expression of long form of PRL receptor normalized to expression of β-actin in ATC1 cells and rat adrenal primary culture. *D*, cAMP levels are not modulated by JAK2 inhibitor. Effects of AG490 on the basal and ACTH induced levels of intracellular cAMP were measured after incubation of ATC1 cells with 0.5 mM isobutyl-1-methylxanthine. Each value represents the mean of six independent experiments ± S.D. *, $p < 0.05$ compared with basal condition.

pg/ml at 5:00 p.m.) indicating that PRL signaling did not affect the basal activity of the pituitary-adrenal axis. To explore a possible direct effect of PRL on adrenal cortex activity, corticosterone production in response to human or ovine recombinant PRL was assayed in primary culture of rat adrenocortical cells and murine adrenocortical cell line (ATC1). PRL treatment did not stimulate corticosterone release by rat adrenocortical primary cells or murine ATC1 cells (Fig. 1*A*) despite expression of PRLR (Fig. 1*B*) in both cell lines.

It has been postulated that ACTH-induced corticosterone production could be enhanced by PRL (10). However, in our experiments addition of PRL (10^{-8} to 10^{-7} M) did not enhance the effect of increasing concentrations of ACTH (10^{-11} to 10^{-9} M) on corticosterone secretion by primary cells from rat adrenals (supplemental Fig. S1). The discrepancy between our observations and published data is likely a result of contamination batches of purified extractive PRL from ovine pituitary (Sigma-Aldrich) with a high concentration of ACTH (supplemental Table S1). These unexpected results were observed on multiple batches of purified PRL. Therefore, data generated with this compound require cautious interpretation.

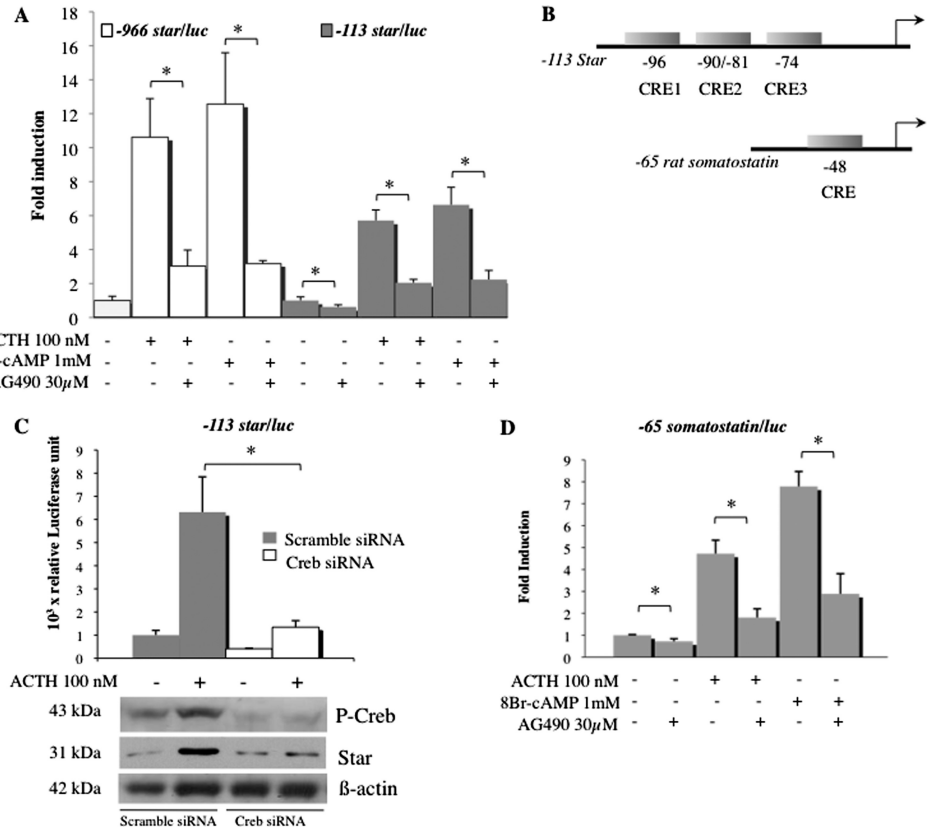

FIGURE 2. **JAK2 modulates basal and cAMP-induced transcriptional activities driven by the murine *Star* or rat somatostatin gene promoters.** *A*, ATC1 cells were transfected with reporter constructs controlled by the 966 bp or 113 bp of the murine wild-type *Star* promoter and were treated or not for 17 h with AG490 in the presence or absence of ACTH or cAMP for the last 16 h. Results are expressed relative to the luciferase activity value under basal culture condition and represent the mean ± S.D. (*error bars*) of three independent experiments performed in quadruplicate (*, $p < 0.05$ compared with ACTH or 8-Br-cAMP (inhibitor-free)-treated cells). *B*, schematic represents 113 proximal bp of the murine *Star* gene promoter and the 65 proximal bp of the rat somatostatin gene. For each construct, *gray boxes* represent CRE displaying a CREB-protein binding activity previously demonstrated (3, 31). *C*, ACTH responsiveness of the −113 *Star* gene promoter is controlled by CREB protein in ATC1 cells. ATC1 cells were transfected with CREB siRNA or Scramble siRNA for 24 h prior to transfection with the −113 *Star/luc* construct and treated or not with ACTH for 16 h prior to harvesting. Results represent three independent experiments performed in triplicate. Data are expressed as means ± S.D. (*, $p < 0.05$ compared with ACTH-treated cells transfected with Scramble siRNA). In the same experiment Western blot analysis was performed on the cellular extracts to confirm specific CREB knockdown by the CREB siRNA and effect on endogenous STAR levels. The blots were incubated with the anti-Ser(P)^{133}-CREB, STAR, and β-actin antibodies. *D*, to test the ability of the JAK2 inhibitor to repress a CREB protein-dependent promoter transcriptional activity, ATC1 cells were transfected with the −65 *somatostatin/luc* construct and treated with ACTH or 8-Br-cAMP for the last 16 h without AG490. Results are expressed relative to the luciferase activity value under basal culture condition and represent the mean ± S.D. of three independent experiments performed in quadruplicate. *, $p < 0.05$ compared with basal or ACTH- or 8-Br-cAMP inhibitor-free-treated cells, respectively.

Although, PRL *per se* had no effect on corticosterone production, treatment with the selective JAK2 inhibitor AG490 significantly reduced both basal and ACTH/cAMP-stimulated corticosterone release by either primary cells or ATC1 cells (Fig. 1, *A* and *C*). In ATC1 cells, AG490 was at least as efficient as the PKA inhibitor H89 in repressing the basal or stimulated corticosterone secretion (Fig. 1*C*). Given the prominent role of the cAMP pathway in controlling adrenal cortex steroidogenesis activity, we wondered whether cAMP levels could be directly modulated through a JAK2-dependent mechanism in ATC1 cells (Fig. 1*D*). Neither basal nor ACTH-induced cAMP levels were affected by AG490 treatment, suggesting that JAK2 contribution was downstream from cAMP production. LH signaling was shown to induce JAK2 phosphorylation in the ovarian follicular cells (30), suggesting that similarly, ACTH could influence phosphorylation of JAK2 as a prerequisite for its functional activity. Therefore, we evaluated JAK2 phosphorylation in response to ACTH stimulation in ATC1 cells and primary adrenal cell culture. As expected, recombinant PRL induced JAK2 phosphorylation in our experiments. However, the JAK2 phosphorylation level remained unaltered by ACTH treatment in both cell types. In our experiments, unlike PRL, ACTH had no effect on JAK2 phosphorylation tested in either primary cultures or the ATC1 line (supplemental Fig. S2). Altogether, these data demonstrate that JAK2 but not PRL is involved in modulation of glucocorticoid production by adrenocortical cells through effects downstream from cAMP production.

JAK2-mediated Steroidogenesis Depends on CREB Factor—STAR is the rate-limiting enzyme of steroid hormone biosynthesis, so we investigated the impact of JAK2 activity on *Star* gene transcription. ATC1 cells were transiently transfected with the luciferase reporter constructs driven by mouse *Star* 5′-flanking regulatory sequences. As expected, transcriptional activities driven by both the 966-bp or the proximal 113-bp promoter constructs were efficiently stimulated by either ACTH or cAMP (Fig. 2*A*). Moreover, basal and ACTH- and cAMP-stimulated activities were all inhibited by AG490. Hence, these data strongly suggested that the effect of JAK2 occurs at a transcriptional level. Functional cooperation between multiple *cis*-elements located in the proximal region of

Star promoter is essential for cAMP responsiveness. These elements include three CRE half-sites able to bind several members of the CREB family (3, 4) (Fig. 2*B*). However, a specific role for CREB in *Star* transcription had not been directly evaluated. To specify the role of CREB, the 113-bp promoter construct was transfected in ATC1 cells together with CREB siRNA duplexes (see "Experimental Procedures"). Transfection of the CREB siRNA inhibited basal and ACTH-induced *Star* proximal promoter activity by 60 and 77%, respectively (Fig. 2*C*). This result demonstrated that CREB was essential to basal and hormonal responsiveness of the *Star* proximal promoter. The molecular mechanisms governing cAMP-induced CREB activities have been studied extensively through its ability to bind and activate the cAMP-response element in the promoter of the neuropeptide somatostatin gene (31, 32). To confirm the role of JAK2 on the transcription of another CREB-dependent gene, ATC1 cells were transfected with the rat somatostatin CRE containing a minimal promoter construct and exposed to AG490 in the presence or absence of ACTH or cAMP. Basal and ACTH- and cAMP-stimulated activities of the somatostatin promoter were repressed by AG490, confirming the effect of JAK2 on CREB-dependent transcription (Fig. 2*D*). Downstream canonical substrates of the JAK2 kinase are STAT proteins. Involvement of STAT5a in JAK2-mediated transcriptional activities of the 113-bp and 966-bp *Star* promoters were analyzed in ATC1 cells by co-transfection of a constitutively active STAT5a (STAT5a ca)-expressing vector (Fig. 3*A*). As expected, activity of the control reporter construct driven by the LHRE containing six STAT5 binding sites was strongly simulated by STAT5a ca. By contrast, no significant variation was observed for transcription of the reporter constructs driven by the *Star* or the somatostatin gene promoters. We concluded that JAK2-mediated CRE-dependent gene transcription did not rely on STAT5a. These data were confirmed by STAT5a silencing analysis (Fig. 3*B*). Indeed, STAT5a knockdown had no effect on the −966-bp *Star* promoter activity in ATC1 cells treated without PRL, whereas it strongly affected PRL responsiveness of the LHRE *tk*/*luc* construct. Previously published data provided evidence that JAK2 was involved in magnolol-induced *Star* gene transcription and acted as the upstream regulator of the MEK-ERK pathway that in turn controlled the phosphorylation of CREB (33). However in our experiments, adrenocortical cell exposure to PD98059 (MAPK inhibitor) had no effect on ACTH-stimulated STAR protein expression (supplemental Fig. S3).

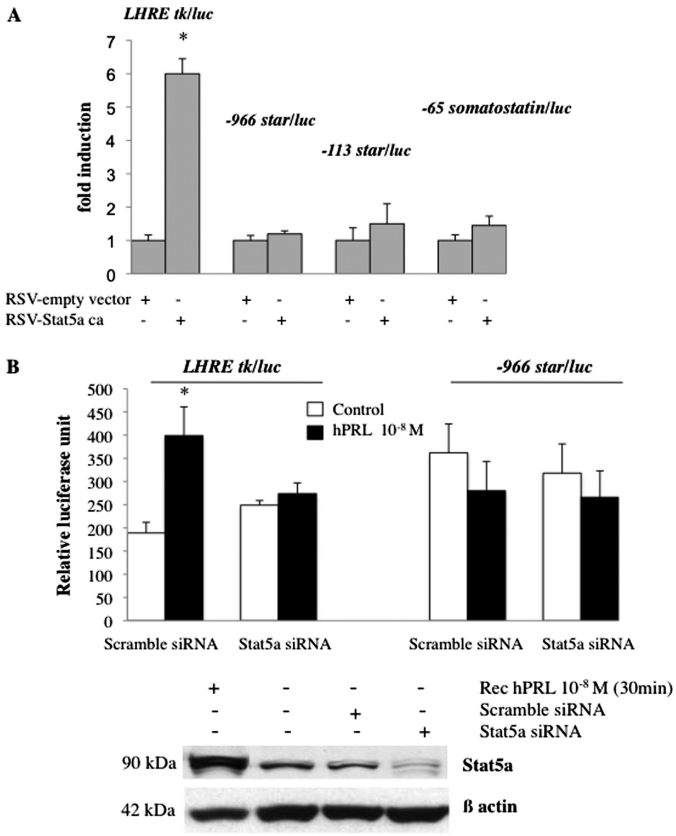

FIGURE 3. **The CREB-dependent STAR or rat somatostatin promoter construct transcriptional activities are not responsive to the STAT5a.** *A*, to examine a direct role of the JAK2/STAT5 pathway on the −65 somatostatin gene −966 and −113 *Star* promoter transcriptional activity, co-transfection experiments in ATC1 cells in basal conditions were performed with either 250 ng of plasmid encoding the constitutively active STAT5a mutant or corresponding pRSV empty vector together with reporter construct. As control, transfection with a reporter construct containing six binding sites for STAT5a associated with the minimal thymidine kinase promoter named LHRE was included in the experiment. After the end of transfection, cells were grown for 24 h in basal medium before being harvested for luciferase assay. Results are expressed relative to the luciferase activity value obtained for transfection of each reporter gene with the RSV empty vector. Each *bar* represents the mean ± S.D. (*error bars*) of six independent experiments performed in quadruplicate. *, $p < 0.05$ compared with transfection with RSV empty vector. *B*, *upper*, the −966 *Star* promoter activity is not controlled by STAT5a in ATC1 cells. ATC1 cells were transfected with STAT5a siRNA or Scramble siRNA 36 h prior to transfection with the −996 *Star/luc* construct and were treated with or without hPRL for 12 h prior to harvesting. Results represent three independent experiments performed in triplicate. Data are expressed as means ± S.D. (*, $p < 0.05$ compared for each reporter construct with PRL-untreated cells transfected with Scramble siRNA). *Lower*, Western blot analysis was performed on ATC1 cell extracts to confirm specific STAT5 knockdown by the STAT5 siRNA.

To confirm directly the specific involvement of JAK2 in adrenocortical steroidogenic activity, we performed knock-down experiments using JAK2 siRNA duplexes (see "Experimental Procedures"). Basal corticosterone release from siRNA-transfected ATC1 cells was decreased by 60% compared with Scramble siRNA-transfected cells (Fig. 4*A*). This was in agreement with data obtained with the pharmacological inhibitor AG490. Interestingly, JAK2 knockdown resulted in a strong reduction of both Ser^{133}-phosphorylated CREB ($Ser(P)^{133}$-CREB) and STAR protein levels and to a lesser extent in total CREB levels (Fig. 4*B*). These data strongly suggested that JAK2 promoted corticosterone production at least by maintaining phospho-CREB levels which in turn stimulated *Star* gene expression.

Recent studies reported a role of JAK2 in protecting proteins against proteasomal degradation (18, 34). This suggested that JAK2 could protect CREB from degradation through a similar mechanism. To test this hypothesis, adrenocortical cells were exposed to AG490 in the presence of either the proteasome inhibitor MG132 or the nuclear export inhibitor leptomycin to prevent proteosomal degradation through nuclear sequestration. In agreement with JAK2 siRNA experiments, AG490 treatment specifically disrupted basal and ACTH-induced $Ser(P)^{133}$-CREB accumulation in both ATC1 cells and primary

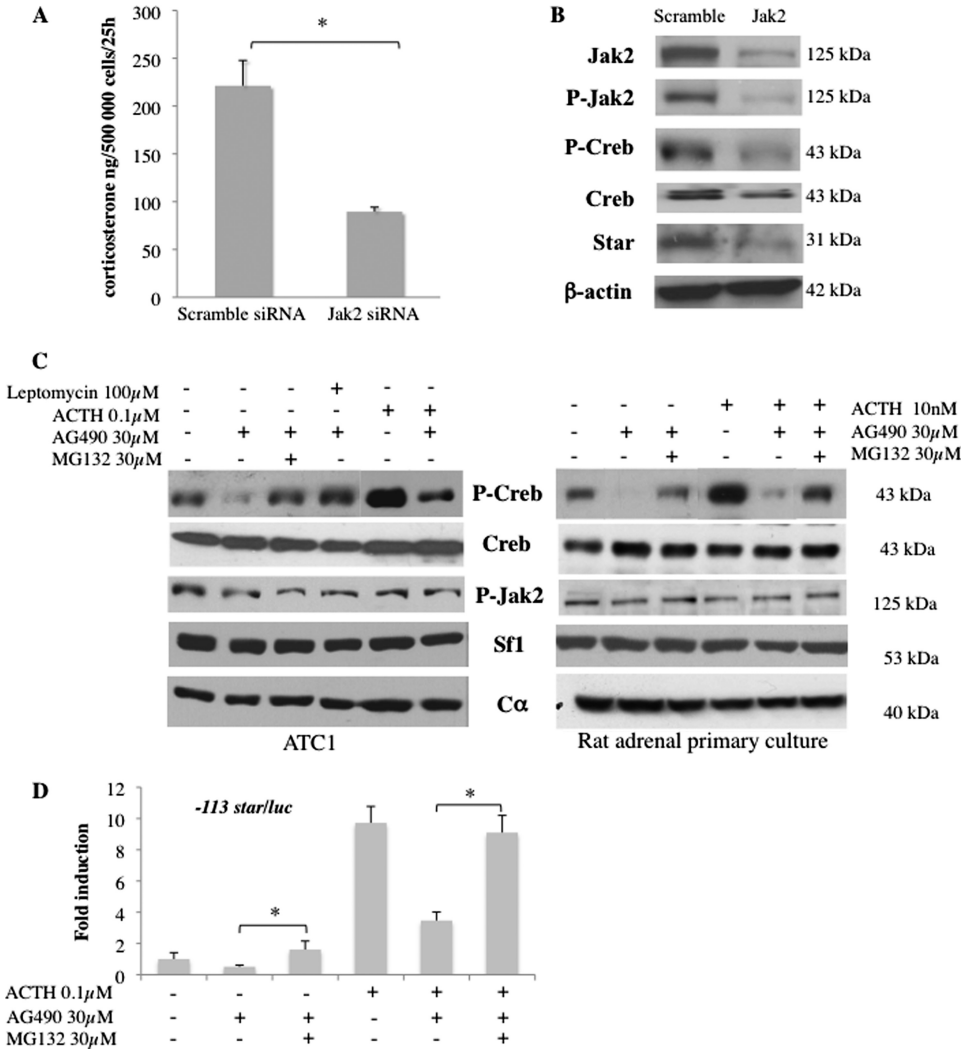

FIGURE 4. **JAK2 mediates basal or ACTH-induced steroidogenesis by preventing phosphorylated CREB from proteosomal degradation.** *A*, basal corticosterone production by ATC1 cells transiently transfected with either Scramble siRNA or JAK2 siRNA and analyzed by RIA. Each *bar* represents the mean ± S.D. (*error bars*) of four independent experiments (*, $p < 0.05$). *B*, Western blot analysis of cellular extracts of ATC1 cells transiently transfected with either Scramble siRNA or JAK2 siRNA as indicated. The blots were incubated with anti-JAK2, anti-phosphorylated JAK2, anti-phosphorylated CREB protein, anti-CREB protein, anti-STAR, and anti-β-actin antibodies. *C*, Western blot analysis of cellular extracts of ATC1 cells or rat adrenal primary culture treated either with the AG490 for 17 h without the proteasome inhibitor MG132 or the nuclear export inhibitor leptomycin B for the last 16 h (ATC1), or with the AG490 for 17 h without MG132 (adrenal primary culture), respectively. Effect of AG490 treatment in adrenal primary culture was also analyzed after ACTH exposure for the last 5 min before harvesting the cells. The blots were incubated with anti-phosphorylated CREB, anti-CREB, anti-phosphorylated JAK2, anti-SF1, and anti-catalytic subunit of the PKA (Cα) antibodies. *D*, MG132 antagonizes the AG490 inhibition of the CREB-dependent transcriptional activity of −113 *Star* construct. ATC1 cells were transfected with the −113 *Star* construct and treated with ACTH for the last 16 h without AG490 or MG132. Results are expressed relative to the luciferase activity value under basal culture condition and represent the mean ± S.D. of three independent experiments performed in quadruplicate. *, $p < 0.05$ compared with AG490-treated cells.

cells although it did not alter total CREB nor the SF1 and the PKA catalytic subunit α (Cα) levels (Fig. 4*C*). As expected, transfection experiments showed that MG132 exposure prevented AG490-mediated repression of the transcriptional activity of the −113 *Star* promoter construct in both basal and ACTH conditions (Fig. 4*D*). Taken together, these data show that JAK2 prevents early degradation of CREB. This allows accumulation of Ser^{133}-phosphorylated CREB, which maintained basal and ACTH induced *Star* transcription.

JAK2 and CREB Interact in the Same Protein Complex—Both MG132 and leptomycin treatments prevented the deleterious effects of AG490 on $Ser(P)^{133}$-CREB levels. This indicated that JAK2 could be involved in nuclear sequestration of CREB thereby preventing its proteosomal degradation. Subcellular protein fractions of ATC1 cells were analyzed by Western blotting. This confirmed the presence of P-JAK2 and CREB in the same nuclear fraction (Fig. 5*A*). Detection of the cytoplasmic scavenger receptor class B type 1 (SR-B1) and the nuclear protein SF1 allowed confirmation of the quality of cell fractionation. Because nuclear JAK2 seemed to be involved in the regulation of CREB stability, we hypothesized that CREB may be phosphorylated on tyrosine residues. Whole cell extracts from ATC1 lines were immunoprecipitated with an anti-CREB antibody and subsequently Western blotted with an anti-Tyr(P) antibody (Fig. 5*B*). These experiments revealed that CREB was actually tyrosine-phosphorylated. Importantly, tyrosine phosphorylation was decreased when the cells were treated with AG490 (Fig. 5*C*). Co-immunoprecipitation experiments fur-

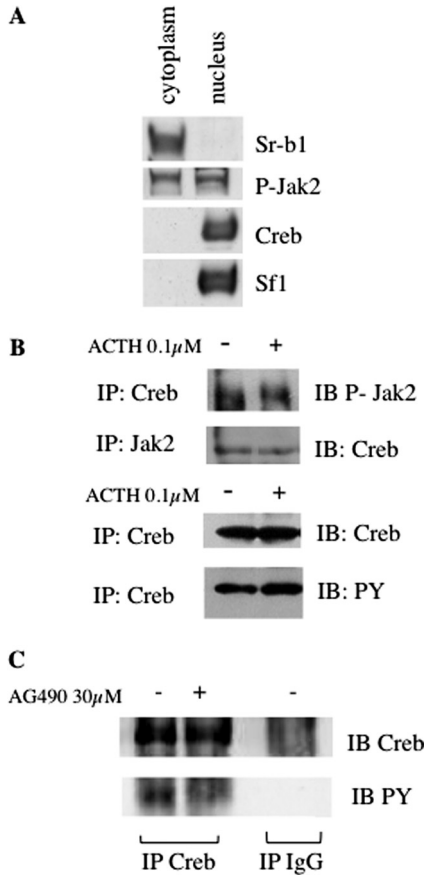

FIGURE 5. **Active JAK2 is found in the nucleus and interacts with CREB protein.** *A*, cytoplasmic and nuclear extracts were prepared from ATC1 cells by cellular fractionation and subjected to Western blot analysis. The blots were incubated with anti-scavenger receptor class B type 1 (*Sr-b1*), anti-CREB, and anti-SF1 primary antibodies. *B*, whole cell extracts of ATC1 cells treated or not with 0.1 μM ACTH for 10 min were immunoprecipitated (*IP*) with JAK2 antibody or CREB antibody and subsequently Western blotted (*IB*) with anti-CREB or anti-JAK2, or anti-Tyr(P) antibodies. *C*, whole cell extracts of ATC1 cells treated or not with 30 μM AG490 for 6 h were immunoprecipitated with CREB antibody or nonimmune rabbit IgG and subsequently Western blotted with CREB or Tyr(P) antibodies.

ther demonstrated that JAK2 and CREB proteins were present in the same protein complex. Taken together, these results suggest that JAK2 tyrosine activity occurs in the same nuclear protein complex as CREB. This interaction may prevent the early proteosomal degradation of the Ser(P)^{133}-CREB isoform, thus allowing its PKA-induced transcriptional activity on target genes.

DISCUSSION

PRL is essential for lactation initiation but is also involved altogether with other hormones in the control of numerous subsequent functions including metabolism, reproduction, and endocrinology (35, 36). The mechanisms by which PRL is implicated in the regulation of the hypothalamo-pituitary-adrenal axis remains to be specified. At the hypothalamic level, it has recently been suggested that PRL exerts dual actions on hypothalamo-pituitary-adrenal axis activity. It directly stimulates ERK/MAPK activity on the corticotropic hormone gene transcription in the paraventricular nucleus and exerts indirect inhibitory actions through modulation of neural pathways to the paraventricular nucleus to modulate plasticity of the neuroendocrine system during lactation (37). Whether PRL acts directly on pituitary ACTH production is controversial because (i) bromocriptine-responsive Cushing disease is rare (38, 39), (ii) hyperprolactinemia and bromocriptine-induced hypoprolactinemia do not influence plasma ACTH levels (40, 41) even though the presence of PRLR on corticotroph cells has been established (42). Furthermore, it is not clear whether the stabilization of ACTH levels by bromocriptine or cabergoline in Nelson syndrome is rather ascribed to an indirect effect of dopaminergic-induced hypoprolactinemia or a direct dopaminergic effect through the abnormal expression of the D2 receptor in some corticotroph tumor cells (43).

Several studies using primary cultures have reported that PRL could act directly on the adrenal gland to enhance glucocorticoid production from adrenals of different mammals, including mouse or human (10–12, 44). Data obtained from *in vivo* pharmacological models are still ambiguous due to difficulties to dissociate direct adrenal responses from those of hypothalamo-pituitary axis to PRL or bromocriptine manipulations. Indeed, its has recently been shown that bromocriptine can directly inhibit steroid production in the adrenal through D2 receptors expressed in the three cortical zones (45, 46).

In the present study, *in vivo* PRL control of adrenal glucocorticoid production was investigated using *Prlr*-deficient mice. Our results showing unaltered basal corticosterone or ACTH plasma levels in this mouse model rule out the possibility that PRL controls basal glucocorticoid production. Production of PRL is increased during stress in rodents (10). A role of PRL in increasing ACTH sensitivity is therefore a reasonable assumption. However, we did not observe alterations of corticosterone and ACTH plasma levels in *Prlr*$^{-/-}$ mice submitted to restraint stress compared with wild-type animals (supplemental Fig. S4). We further attempted to reveal hypothetical PRL-mediated adrenal responses using rat adrenal primary cultures or mouse adrenal cell lines. Although recombinant PRL (10^{-7} to 10^{-8} M) was able to activate the JAK2/STAT5-coupled PRLR signaling pathway both in primary adrenal cells and mouse ATC cells, it did not influence basal and ACTH-induced corticosterone production or *Star* gene transcription. To our surprise, initial experiments with ovine pituitary-purified PRL showed that this hormone was as efficient as ACTH in stimulating corticosterone release (47). Therefore, we analyzed ACTH concentration in several batches of purified PRL. These experiments showed that all batches of ovine PRL were highly contaminated with ACTH. This presumably reflects inappropriate separation and purification of hormonal products from the pituitary. It is therefore important to exercise care when interpreting data generated through the use of ovine purified PRL. Altogether, these results showed that PRL does not exert a direct control on corticosterone production in rodents.

Surprisingly, analysis of corticosterone production by isolated adrenal cells provided clear evidence that unlike PRL, JAK2 was involved in basal and ACTH-induced steroidogenesis. Previous data have already reported an early role of JAK2 in the mediation of hormone effects through action on G protein-coupled receptors including angiotensin receptor AGTR 1a in adrenocortical cells (19) or LH receptor LHCGR in ovarian cells (30). In follicular cells, JAK2 tyrosine phosphorylation was increased in response to LH. In this case, JAK2 involvement was

mostly associated with hormonally induced signaling pathways through a cross-talk between JAK2/STAT5 and the PI3K/Akt pathways, although there was also an association with LH-activated MAPK pathways.

In adrenocortical cells, JAK2 activation is critical for both angiotensin II and magnolol-stimulated steroidogenesis and *Star* expression. This effect of JAK2 is in part mediated through the MAPK/ERK1/2 pathway (19, 33) even though the molecular target of JAK2 was not clearly identified in adrenal cells. Our data rather support the finding that in adrenocortical cells, activated JAK2 levels are not sensitive to ACTH. They exert an essential control on basal and ACTH-induced steroidogenesis by interacting with the PKA through the modulation of CREB protein accumulation. In erythroid cell lines, connections between the cAMP/PKA/CREB and JAK/STAT5 pathways had already been reported in the mediation of prostaglandin E2 and erythropoietin signaling. In this system prostaglandin E2-induced CREB phosphorylation facilitated CBP/P300 recruitment and interaction with STAT5. This in turn resulted in increased STAT5-dependent transcriptional activity (48).

In the present report, we show that CREB-induced *Star* transcription is dependent on JAK2 but is independent of STAT5. Several STAT5-independent strategies are available to allow control of nuclear proteins by JAK2. Recent data show that in addition to controlling cyclin D1 gene expression in proliferative mammary epithelial cells, JAK2 signaling regulates the accumulation of the cyclin D1 protein in the nucleus by inhibiting signal transducers that mediate the phosphorylation (*i.e.* Akt/PKB and GSK-3β) and nuclear export of cyclin D1 (49).

Although initially controversial, the nuclear localization of active JAK2 has been demonstrated by recent studies (18, 50, 51). In mammary epithelial cells, nuclear JAK2 activity had been shown to regulate the amount of transcription factor nuclear factor 1-C2 by preventing its association with and subsequent degradation by the proteasome (18). Using pharmacological inhibitors and specific JAK2 siRNA strategies, we demonstrate that in adrenocortical cells JAK2 acts in the nucleus to stabilize the $Ser(P)^{133}$-CREB by preventing its early proteasomal degradation. This, in turn, allows stimulation of *Star* gene transcription. We propose that the mechanisms underlying this action of JAK2 involve tyrosine phosphorylation of CREB which occurs in a CREB-JAK2 protein complex. Whether physical interactions between JAK2 and CREB are direct or indirect remains to be further determined.

In summary, our data demonstrate for the first time that STAT5-independent nuclear JAK2 signaling plays an essential role in adrenal steroidogenesis by increasing stability of the transcriptionally active CREB protein thus allowing expression of CREB steroidogenic target genes.

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